REMARKS

In the Office Action, claim 297 was objected to for introducing new matter into the disclosure. Claim 297 was also objected to on the basis of the informality "Tris-Cl". Claims 225-244, 265-284, and 297 were rejected under 35 USC 112, first paragraph as lacking enablement. Claims 275 and 297 were rejected under 35 USC 112, second paragraph as indefinite. Claims 215-244 and 255-264 were allowed. The withdrawal of claims 189-214 and 285-290 from consideration has been separately petitioned.

The objections to claim 297

Claim 297 was objected to for allegedly introducing new matter. This objection is traversed. As set forth in the previous response, the hybridization language recited in this claim finds support in Example 3 at page 49 lines 18-23. It is undisputable that PCR involves a hybridization step, and that Example 3 recites a hybridization step with the conditions recited in claim 297. It is also indisputable that Example 3 describes a nucleic acid that hybridizes preferentially to the complement of the sequence depicted in SEQ ID NO: 1 under those conditions. PCR requires preferential hybridization by the primers to each of the complementary strands produced from the target for amplification to occur. Example 3 shows amplification of SEQ ID NO: 1 occurring under the conditions recited in claim 297. No new matter has been added.

If the Examiner is alleging that PCR does not contain a hybridization step, he is invited to clearly state this on the record and to provide evidentiary support for this statement. There are no scientific grounds for this statement. Applicant is only required to challenge the inaccuracy of a scientific assertion by the Examiner to overcome an unsupported rejection, and is then entitled to evidentiary support for the assertion. If no evidentiary support is provided, the rejection remains overcome.

Claim 297 was also objected to on the basis of the informality of "Tris-Cl". This objection is traversed. No legal basis for this objection was stated, and therefore no grounds for objection have been established. Furthermore, "Tris-Cl" is a term used and understood by persons of skill in the art. Attached as Exhibit A are the results of a search of the term "Tris-

CI" in the PubMed database from the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health. 84 references were identified as containing this search term.

Withdrawal of the objections to claim 297 is requested.

The enablement rejection

Claims 225-244, 265-284, and 297 were rejected under 35 USC 112, first paragraph as lacking enablement. This rejection is traversed.

Applicants did not argue in their last response that it is "eronious" to make an enablement rejection based on only one of the Wands factors (although it is); Applicants simply quoted the Training Materials as to the requirements for establishing an enablement rejection. Furthermore, the first Office Action on the merits involved claim language no longer present. No analysis of each of the Wands factors has been provided for the pending claims. An enablement rejection requires factual evidence regarding each of the elements; assertion that the scope is "enormous" does not meet the legal standard.

The enablement rejection contains the following unsupported assertions:

(a) that Applicants have not presented evidence or arguments to establish enablement; they have, and provide additional evidence and arguments here. This is simply boilerplate language.

(b) that no teaching of how to redesign 20% of the amino acids is shown; this is discussed below.

(c) that PCR conditions tend to enhance the "hybrdization" of a probe to a potential gene. Evidentiary support for this statement is requested. Successful PCR conditions, as shown in Example 3 and recited in the claims, are highly selective, amplifying only the intended sequence, otherwise every sequence in the mix would amplify. Selectivity is the entire reason PCR is so valuable.

(d) that 15 mM ammonium sulphate is a high ionic strength. Evidentiary support is requested.

The Office Action statement that "[t]here is no such a hybrdization condition known in the prior art and, if it exists, it would be a very low hybrdization conditions" (page 3, last sentence of second full paragraph) is unclear and unsupported. What is "a very low hybrdization conditions?" This is not a term of art.

Additionally, 15 mM ammonium sulphate is not high ionic strength. An example of a hybridization solution is shown in Exhibit A, using 6x SSC. The salt concentration of 1x SSC is 150 mM sodium chloride; 6x SSC is 900 mM salt. 15 mM ammonium sulphate is not a high salt concentration. This grounds of rejection is completely baseless.

Applicants have disclosed many methods for generating mutants of the disclosed sequences. The previous Office Action (page 3, last paragraph) acknowledged that "mutation methods of couple of residues are taught in the specification and are well known in the prior art." It does not require undue experimentation to provide these teachings to obtain a polypeptide with 80% homology to the disclosed sequence. Scaling up a disclosed invention using techniques admitted to be taught in the application is not undue experimentation.

Applicants teach much more than this, however. Applicants have provided specific direction to one of skill in the art as to which amino acids in the sequence can be altered. Example 2 at pages 47-48 discusses an alignment of delta-six desaturases, including the polypeptide at issue here along with borage and other delta-six desaturases known in the art. This teaching provides extensive specific information to one of skill as to what positions can be substituted and what types of changes can be made while still maintaining delta-6 desaturase activity. There is more than a 20% difference between the aligned delta-6 sequences, providing a sufficient number of regions and positions to test (via point mutagenesis or cassette mutagenesis as taught in the application) to obtain a sequence with 80% homology and still have delta-6 activity. Following these teachings, one of skill could prepare an 80% homologous sequence with specific activity in at most a few months by routinely testing the positions shown as accepting substitution. "[A] considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed."

In re Wands, 858 F.2d at 737, 8 USPQ2d at 1404 (citing *In re Angstadt*, 537 F.2d 489, 502-04, 190 USPQ 214, 218 (CCPA 1976)).

Applicants have provided extensive guidance to one of skill as to how to make the claimed invention. Only routine testing of the positions and substitutions identified in Applicants' alignment is needed to obtain an active sequence with 80% homology.

The grounds stated regarding the hybridization claims also lack support.. Hybridization conditions were known in the art at the time of invention, including in other laboratories for hybridizing to *Mortierella* libraries. *See* Exhibit B, which shows that another laboratory was simultaneously isolating a different gene from another *Mortierella* species via hybridization. Hybridization was described generically as here, because it is a routine technique well known in the art at the time of invention. If the editors had thought there was any lack of clarity in the authors cursory description of their hybridization method, they would have required additional information to be provided. They did not, because hybridization is a routine technique well known in the art, with stock formulas well established, and requiring only minimal experimentation to test the full range of hybridization conditions, which involve varying salt concentration and temperature. Applicants are not required to teach what is well known, as hybridization methods were at the time of invention. There is nothing more basic and well known in molecular biology than how to hybridize nucleic acid sequences.

Accordingly, the presented claims are asserted to be fully enabled, and the application teaches one of skill in the art how to make and use the full scope of the invention. Withdrawal of the enablement rejection is requested.

<u>The rejections under 35 U.S.C. §112, second paragraph</u>

Claims 275 and 297 were rejected under 35 USC 112, second paragraph as indefinite.

(a) Claim 275 and dependent claims 276-284 were rejected as indefinite for the phrase "hybridizes preferentially to the complement of the sequence depicted in SEQ ID NO: 1 under hybridization conditions suitable for sequencing said complement." The arguments set forth in Applicants' previous response are incorporated by reference.

The Office Action asserted that "nucleic acid are known to hybridize to any other nucleic acid sequence under different conditions." Page 4, first paragraph. No scientific basis for this statement has been provided, and Applicants submit that this is not true and request evidentiary support for this statement as they are entitled to do. Furthermore, this is not even the claim language. The claim recites "hybridizes preferentially," which precludes random hybridization to any sequence, covering related sequences sufficiently homologous to hybridize to SEQ ID NO: 1 under conditions suitable for sequencing.

This may explain the incorrect statement that Applicants did not respond to the previous Office Action. Claim 275 was in fact amended to require preferential hybridization; see pages 14-15 of the previous response. The acceptability of this exact language by the PTO was also recited (Training Materials For Examining Patent Applications With Respect To 35 U.S.C. Section 112, First Paragraph-Enablement Chemical/Biotechnical Applications at Example A: Analysis). This argument has not been addressed.

Most significantly, the statement that one of ordinary skill would not understand the phrase "hybridization conditions suitable for sequencing" the complement is simply false. Nucleic acid sequencing had been performed for decades before this application was filed, hybridization conditions suitable for sequencing were known in the art, sequencing kits were widely available from a number of manufacturers, and sequencing of nucleic acid is also described in the application. Applicants are entitled to use language of their choosing to claim their invention, including functional language; there is no particular language required. Applicants further request evidentiary support for the statement that one of skill in the art does not understand what "hybridization conditions suitable for sequencing" are.

As no evidence has been provided to support the rejections of claim 275-284, indefiniteness has not been established. Withdrawal of the rejection is requested.

(c) Claim 297 was rejected as indefinite and confusing for the phrase "nucleic acid hybridizes preferentially to the complement of the sequence depicted in SEQ ID NO: 1 at 50 degrees Celsius in 60 mM Tris-Cl (pH 8.5) and 15 mM $(NH_4)_2SO_4$ and 2 mM $MgCl_2$." This very specific phrase was said to not clearly set forth the metes and bounds of the patent

protection desired. This rejection is traversed. It is difficult to imagine how the claim could be more definite in its scope.

If the Examiner is alleging that PCR does not contain a hybridization step, he is invited to clearly state this on the record and to provide evidentiary support for this statement. Again, there are no scientific grounds for this statement. Applicant is only required to challenge the inaccuracy of a scientific assertion by the Examiner to overcome an unsupported rejection, and is then entitled to evidentiary support for the assertion. If no evidentiary support is provided, the rejection remains overcome.

To the extent that "[f]or examination purposes only, the conditions is eliminated from the claim" (page 4 of the Office Action) by the Examiner, the Examiner is not entitled to amend the claims to remove the language, but is required to examine the claims as presented.

Indefiniteness of claim 297 therefore has not been established, as no grounds for this rejection have been provided. The Examiner has examined a claim that does not exist by making an unauthorized amendment. The claim is clear and definite. Withdrawal of the rejection of claim 297 is requested.

CONCLUSION

Applicants request reconsideration of the claims in view of the above amendments and remarks. A notice of allowance is earnestly solicited. If a telephone conference would expedite allowance of this matter, the Examiner is welcome to contact the undersigned.

If an appropriate payment does not accompany or precede this submission, the Commissioner is hereby authorized to charge any required fees, including any petition for extension of time, or to credit any overpayment, to Deposit Account No. 50-2518, billing reference no. 15611-7032 (7000934001).

Respectfully submitted,

By: _____

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EXHIBIT A

1

Molecular Cloning

A LABORATORY MANUAL

SECOND EDITION

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Cold Spring Harbor Laboratory Press
1989

Hybridization of Radiolabeled Probes to Nucleic Acids Immobilized on Nitrocellulose Filters or Nylon Membranes

Although the method given below deals with RNA or DNA immobilized on nitrocellulose filters, only slight modifications are required to adapt the procedure to nylon membranes. These modifications are noted at the appropriate places in the text.

1. Prepare the prehybridization solution appropriate for the task at hand. Approximately 0.2 ml of prehybridization solution will be required for each square centimeter of nitrocellulose filter or nylon membrane.

   The prehybridization solution should be filtered through a 0.45-micron disposable cellulose acetate filter (Schleicher and Schuell Uniflow syringe filter No. 57240 or equivalent).

Prehybridization solutions

For detection of low-abundance sequences:

Either
6 × SSC (or 6 × SSPE)
5 × Denhardt's reagent
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA

or
6 × SSC (or 6 × SSPE)
5 × Denhardt's reagent
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA
50% formamide

For preparation of Denhardt's reagent and denatured, fragmented salmon sperm DNA, see Table 9.1.

Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by stirring on a magnetic stirrer with Dowex XG8 mixed-bed resin for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

For detection of moderate- or high-abundance sequences:

Either
6 × SSC (or 6 × SSPE)
0.05 × BLOTTO

or
6 × SSC (or 6 × SSPE)
0.05 × BLOTTO
50% formamide

For preparation of BLOTTO, see Table 9.1.

When ^{32}P-labeled cDNA or RNA is used as a probe, poly(A)$^+$ RNA at a concentration of 1 μg/ml may be included in the prehybridization and hybridization solutions to prevent the probe from binding to T-rich sequences that are found fairly commonly in eukaryotic DNA.

2. Float the nitrocellulose filter or nylon membrane containing the target DNA on the surface of a tray of 6 × SSC (or 6 × SSPE) until it becomes thoroughly wetted from beneath. Submerge the filter for 2 minutes.

3. Slip the wet filter into a heat-sealable bag (e.g., Sears Seal-A-Meal or equivalent). Add 0.2 ml of prehybridization solution for each square centimeter of nitrocellulose filter or nylon membrane.

   Squeeze as much air as possible from the bag. Seal the open end of the bag with the heat sealer. Incubate the bag for 1–2 hours submerged at the appropriate temperature (68°C for aqueous solvents; 42°C for solvents containing 50% formamide).

   Often, small bubbles of air form on the surface of the filter as the temperature of the prehybridization solution increases. It is important that these bubbles be removed by occasionally agitating the fluid in the bag; otherwise, the components of the prehybridization solution will not be able to coat the filter evenly. This problem can be minimized by heating the prehybridization solution to the appropriate temperature before adding it to the bag.

4. If the radiolabeled probe is double-stranded, denature it by heating for 5 minutes at 100°C. Single-stranded probe need not be denatured. Chill the probe rapidly in ice water.

   Alternatively, the probe may be denatured by adding 0.1 volume of 3 N NaOH. After 5 minutes at room temperature, transfer the probe to ice water and add 0.05 volume of 1 M Tris · Cl (pH 7.2) and 0.1 volume of 3 N HCl. Store the probe in ice water until it is needed.

   For Southern hybridization of mammalian genomic DNA where each lane of the gel contains 10 μg of DNA, 10–20 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/μg or greater) should be used. For Southern hybridization of fragments of cloned DNA where each band of the restriction digest contains 10 ng of DNA or more, much less probe is required. Typically, hybridization is carried out for 6–8 hours using 1–2 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/μg or greater).

5. Working quickly, remove the bag containing the filter from the water bath. Open the bag by cutting off one corner with scissors. Add the denatured probe to the prehybridization solution, and then squeeze as much air as possible from the bag. Reseal the bag with the heat sealer so that as few bubbles as possible are trapped in the bag. To avoid radioactive contamination of the water bath, the resealed bag should be sealed inside a second, noncontaminated bag.

   When using nylon membranes, the prehybridization solution should be *completely* removed from the bag and immediately replaced with hybridization solution. The probe is then added and the bag is resealed.

Hybridization solution for nylon membranes

6 × SSC (or 6 × SSPE)
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA
50% formamide (if hybridization is to be carried out at 42°C)

6. Incubate the bag submerged in a water bath set at the appropriate temperature for the required period of hybridization.

7. Wearing gloves, remove the bag from the water bath and immediately cut off one corner. Pour out the hybridization solution into a container suitable for disposal, and then cut the bag along the length of three sides. Remove the filter and immediately submerge it in a tray containing several hundred milliliters of 2 × SSC and 0.5% SDS at room temperature.

   Important: Do not allow the filter to dry out at any stage during the washing procedure.

8. After 5 minutes, transfer the filter to a fresh tray containing several hundred milliliters of 2 × SSC and 0.1% SDS and incubate for 15 minutes at room temperature with occasional gentle agitation.

   If short oligonucleotides are used as probes, washing should be carried out only for brief periods (1–2 minutes) at the appropriate temperature. For a discussion of the stability of hybrids involving oligonucleotides, see Chapter 11.

9. Transfer the filter to a flat-bottom plastic box containing several hundred milliliters of fresh 0.1 × SSC and 0.5% SDS. Incubate the filter for 30 minutes to 1 hour at 37°C with gentle agitation.

10. Replace the solution with fresh 0.1 × SSC and 0.5% SDS, and transfer the box to a water bath set at 68°C for an equal period of time. Monitor the amount of radioactivity on the filter using a hand-held minimonitor. The parts of the filter that do not contain DNA should not emit a detectable signal. You should not expect to pick up a signal on the minimonitor from filters containing mammalian DNA that has been hybridized to single-copy probes.

11. Briefly wash the filter with 0.1 × SSC at room temperature. Remove most of the liquid from the filter by placing it on a pad of paper towels.

12. Place the damp filter on a sheet of Saran Wrap. Apply adhesive dot labels marked with radioactive ink to several asymmetric locations on the Saran Wrap. These markers serve to align the autoradiograph with the filter. Cover the labels with Scotch Tape. This prevents contamination of the film holder or intensifying screen with the radioactive ink.

    Radioactive ink is made by mixing a small amount of ^{32}P with waterproof black drawing ink. We find it convenient to make the ink in three grades: very hot

EXHIBIT B

Biosci. Biotech. Biochem., 61 (4), 592–598, 1997

Purification, Characterization, and cDNA Cloning of a Novel α-Galactosidase from *Mortierella vinacea*

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A novel α-galactosidase, designated α-galactosidase II, was isolated from the culture filtrate of *Mortierella vinacea*. The molecular size of the purified enzyme estimated by gel filtration was 60 kDa, which agreed with that, 51–62 kDa, estimated by SDS–PAGE. The enzyme was thermolabile at neutral pH, but the addition of BSA to the enzyme solution at the concentration of 0.01% increased its stability considerably. The enzyme appears to be novel because it showed a distinct substrate specificity from other microbial α-galactosidases on galactomanno-oligosaccharides, prepared from galactomannan, that is, the enzyme liberated not only side-chain α-galactosyl residue from 6^3-mono-α-D-galactopyranosyl-β-1,4-D-mannotetraose but also terminal α-galactosyl residue from 6^3-mono-α-D-galactopyranosyl-β-1,4-D-mannotriose. In addition, the enzyme acted on galactomannans effectively. α-Galactosidase II cDNA was cloned and its nucleotides sequenced. The deduced amino acid sequence showed that the mature enzyme consisted of 376 amino acid residues with a molecular mass of 41,334 Da. The derived amino acid sequence of the enzyme showed 31–49% sequence similarity with those of α-galactosidases from other origins.

Key words: α-galactosidase; *Mortierella vinacea*; cDNA cloning; galactomanno-oligosaccharides; specificity of α-galactosidase

α-Galactosidases (α-D-galactoside galactohydrolase, EC 3.2.1.22) are known to occur widely in microorganisms, plants, and animals and some of them have been purified and characterized.[1] α-Galactosidase from *Mortierella vinacea*, probably identical to α-galactosidase I in this study, has been purified, crystallized, and characterized.[2] The enzyme was reported to be stable at neutral and alkaline pH and to hydrolyze *p*-nitrophenyl-α-D-galactopyranoside, melibiose, raffinose, and stachyose.

Raffinose is widely distributed in the plant kingdom, especially in the sugar beet. The content of raffinose in the sugar beet gradually increases during storage.[3] In the beet sugar industry, raffinose is known as an obstacle to the normal crystallization of beet sugar. If raffinose in beet molasses can be removed by enzymatic treatment, it is possible to improve the crystallization efficiency of beet sugar.[3] For this purpose, *M. vinacea* α-galactosidase has been used in the beet sugar refining process in Japan.

There are many studies on α-galactosidases from microorganisms, but only a few on the substrate specificity of the enzyme. Moreover, the study of the specificity has been done by using such oligosaccharides as *p*NP-Gal, melibiose, raffinose, and stachyose.

We have prepared some kinds of galactomanno-oligosaccharides, such as Gal^3Man_3 and Gal^3Man_4, from the hydrolyzate of galactomannan with β-mannanase to investigate substrate specificity of α-galactosidases on these galactomanno-oligosaccharides.[4,5] Gal^3Man_3 has an α-galactosyl residue (designated the terminal α-galactosyl residue) attached to the C-6 position of the non-reducing end mannose of β-1,4-mannotriose, while Gal^3Man_4 has an α-galactosyl residue (designated the side-chain α-galactosyl residue) attached to the C-6 position of the third mannose from the reducing end of β-1,4-mannotetraose.

Microbial α-galactosidases were classified into two groups based on the substrate specificity on these galactomanno-oligosaccharides.[6,7] Enzymes belonging to each group liberated either the terminal α-galactosyl residue from Gal^3Man_3 or the side-chain α-galactosyl residue from Gal^3Man_4. It can be said that *M. vinacea* α-galactosidase II purified in this study is a novel enzyme because it acted on both substrates.

Complementary and genomic DNAs encoding α-galactosidase have been cloned from various sources including human,[8] guar,[9] yeast,[10] *Aspergillus niger*,[11] coffee bean,[12] *E. coli*,[13] and *Streptococcus mutans*.[14] In addition, the cDNA for α-galactosidase I from *M. vinacea* has also been cloned and its primary structure analyzed.[15]

Here we report the purification and characterization of α-galactosidase II from *M. vinacea*, and its primary structure from the nucleotide sequence of the cDNA.

Materials and Methods

p-Nitrophenyl-α-D-galactopyranoside (*p*NP-Gal) was purchased from Nacalai Tesque, Inc. DEAE-Sephadex A-50, Sephacryl S-300 HR, and Superdex 200 HR were from Pharmacia Biotech and SE-cellulose (SE53)

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Abbreviations: PVDF, polyvinylidene difluoride; CBB, Coomassie Brilliant Blue; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; *pI*, isoelectric point; *p*NP-Gal, *p*-nitrophenyl-α-D-galactopyranoside; BSA, bovine serum albumin; Gal^3Man_3, 6^3-mono-α-D-galactopyranosyl-β-1,4-D-mannotriose; Gal^3Man_4, 6^3-mono-α-D-galactopyranosyl-β-1,4-D-mannotetraose; Endo H, endo-β-*N*-acetylglucosaminidase H.

was from Whatman. [^{32}P] α-dCTP, a cDNA synthesis kit, and a cDNA cloning kit (λgt10) were obtained from Amersham.

Microorganisms and culture conditions. The strain of *Mortierella vinacea* used throughout this work was kindly donated by the Hokkaido Sugar Co. *M. vinacea* was cultured as described previously.[15]

Purification of α-galactosidase. All purification procedures were done at 4°C. The culture filtrate was brought to 90% saturation by adding $(NH_4)_2SO_4$. The precipitate was collected by centrifugation, dissolved in the minimum amount of water, and dialyzed against 10 mM sodium phosphate, pH 7.0. After the removal of insoluble materials by centrifugation, the dialyzed enzyme solution was put on a column (3 × 20 cm) of DEAE-Sephadex A-50, equilibrated with the same buffer. α-Galactosidase activity passed through the column was collected and dialyzed against 10 mM sodium acetate, pH 5.0, and put on a column (1.6 × 25 cm) of SE 53 equilibrated with the same buffer. The α-galactosidase was eluted with a linear gradient of 0 to 0.5 M NaCl in the same buffer. Active fractions were collected and the concentrated enzyme solution was put on a column (2.2 × 87 cm) of Sephacryl S-300 HR equilibrated with 10 mM sodium acetate, pH 5.0, containing 150 mM NaCl. Active fractions were collected and stored at 4°C.

Assay of α-galactosidase activity. α-Galactosidase activity was assayed by measuring the amount of *p*-nitrophenol released from *p*NP-Gal by the incubation at pH 5.2 and 40°C for 10 min as described by Suzuki *et al.*[2] The reaction mixture contained 0.1 ml of 10 mM *p*NP-Gal, 0.8 ml of 0.1 M sodium acetate, pH 5.2, and 0.1 ml of enzyme solution. The reaction was stopped by adding 1.0 ml of 0.2 M sodium carbonate. The amount of *p*-nitrophenol released was measured by the absorbance at 400 nm. One unit of the enzyme activity was defined as the amount of enzyme which released 1 μmol of *p*-nitrophenol from *p*NP-Gal per min at pH 5.2 and 40°C.

Measurement of protein. The distribution of protein in the purification steps was measured by the absorbance at 280 nm, assuming that the absorbance at the concentration of 1 mg protein/ml is 1.0.

Preparation of galactomanno-oligosaccharides. Galactomanno-oligosaccharide having α-1,6-galactosyl side chain on β-1,4-mannotetraose, Gal^3Man_4, was prepared from a hydrolyzate of copra galactomannan by using *Streptomyces* β-mannanase.[4] In addition, galactomanno-oligosaccharide with a terminal galactose at the non-reducing end of β-1,4-mannotriose, Gal^3Man_3, was prepared from Gal^3Man_4 by cutting off the non-reducing end mannosyl residue of the saccharide with *Aspergillus niger* β-mannosidase.[5] The structures of Gal^3Man_3 and Gal^3Man_4 are shown in Fig. 1.

Substrate specificity. Hydrolyses of galacto-oligosaccharides such as melibiose, raffinose, and stachyose, and of galactomanno-oligosaccharides, such as Gal^3Man_3 and Gal^3Man_4, by the purified α-galactosidase II, were done at pH 4.0 (McIlvaine buffer[16]) and 30°C. The sugar sample after enzyme reaction was analyzed by TLC (Silica gel 60, Merck) for the characterization of the hydrolysis products. The reaction products were developed with 1-propanol–nitromethane–water (5:2:3, v/v). The sugars on the plate were detected by heating at 140°C for 5 min after spraying with sulfuric acid.

The extent of hydrolysis of galactomannan was analyzed by the Somogyi–Nelson technique[17] using D-galactose as a standard and the releasing of galactose was confirmed by TLC. After prolonged incubation with α-galactosidase II, the sugar composition of guar gum was analyzed. Enzyme-treated guar gum was recovered by precipitation with ethanol and washed further with 70% ethanol. After drying, 6 mg of the sample was dissolved in 1 ml of 2 M H_2SO_4 and hydrolyzed at 100°C for 3 h, followed by neutralization with $BaCO_3$. The alditol acetate derivatives of the glycosyl residues of the sample were analyzed by gas-liquid chromatography with an ECNSS-M column (0.3 × 150 cm) at 180°C.[18]

Electrophoretic analysis. SDS-PAGE was done as described by Laemmli.[19] The isoelectric point of the purified enzyme was measured by isoelectric focusing with the PhastSystem (Pharmacia Biotech). The protein in the gel were stained with CBB R-250 and *N*-linked oligosaccharide chains were detected with Con A-peroxidase conjugate after blotting on to a PVDF membrane using a semi-dry blotting system (Trans-blot, Bio-Rad Laboratories).

N-Terminal amino acid sequencing. After the protein in the gel was blotted on a PVDF membrane, the membrane was stained with CBB R-250 to detect protein. The protein band was cut out and put on a gas phase protein sequencer (477A, Applied Biosystems Inc.).

RNA isolation. Total RNA was prepared from mycelia by the phenol-chloroform method[20] and poly(A$^+$)RNA was purified with an oligo (dT)-cellulose column (Pharmacia Biotech).

Complementary DNA cloning and nucleotide sequencing. A cDNA library in λgt10 was constructed and clones having α-galactosidase II cDNA were screened for with a synthetic oligonucleotide probe deduced from the *N*-terminal amino acid sequence of α-galactosidase II. An insert DNA was subcloned into pUC19 and was sequenced by the dideoxy chain termination method.[21]

Results

Purification of α-galactosidase

By DEAE-Sephadex A-50 column chromatography, α-galactosidase activity was separated into two fractions. The adsorbed fraction was designated as α-galactosidase I and the fraction passed through the column was designated α-galactosidase II. The α-galactosidase II fraction was separated by ion exchange chromatography on an SE-cellulose column and gel filtration on a Sephacryl S-300 HR column. In the gel filtration chromatography α-galactosidase II ap-

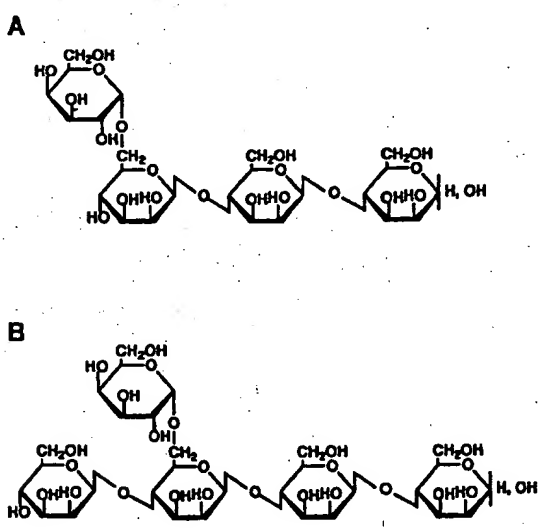

Fig. 1. Structures of Galactomanno-oligosaccharides.
A, Gal^3Man_3 (having terminal α-galactosyl residue); B, Gal^3Man_4 (having side-chain α-galactosyl residue).

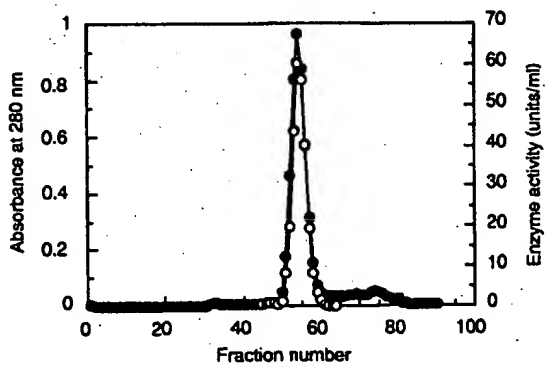

Fig. 2. Gel Filtration of α-Galactosidase II on Sephacryl S-300 HR.
The flow rate was 40 ml/h and 4.0 ml fractions were collected. ●, absorbance at 280 nm; ○, α-galactosidase activity.

Table I. Summary of Purification Steps of α-Galactosidase II

Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
Culture filtrate	5,260	20,800	0.25	100	1.0
$(NH_4)_2SO_4$ fractionation	3,960	1,100	3.60	75.3	14.2
DEAE-Sephadex A-50	1,200	72.3	16.6	22.8	65.6
SE-cellulose	948	28.6	32.0	18.0	126
Sephacryl S-300 HR	929	15.6	59.6	17.7	236

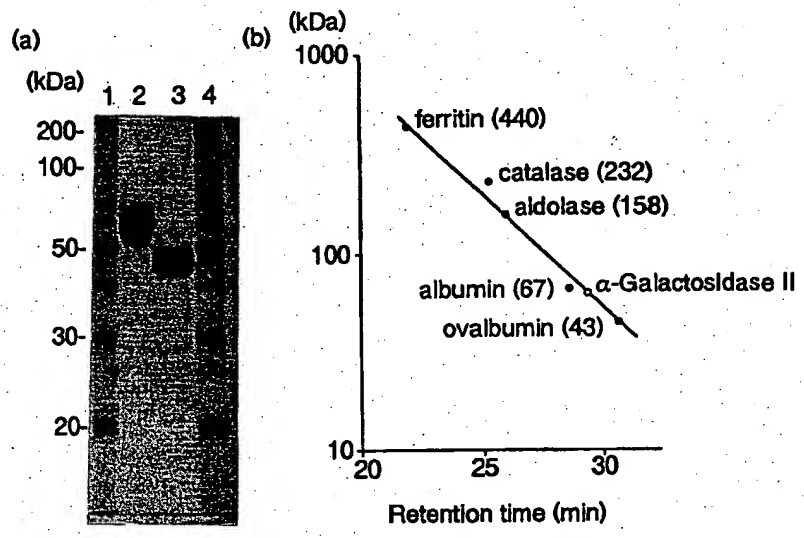

Fig. 3. Homogeneity and Molecular Mass Measurements of α-Galactosidase II by SDS-PAGE (a) and Gel Filtration (b).

(a) The enzyme (20 μg) was electrophoresed on 10.0% acrylamide gel and stained with CBB R-250. Lane 1, molecular size marker; lane 2, α-galactosidase II; lane 3, α-galactosidase II digested with 2 mU of Endo H at pH 5.3 and 37°C for 16 h.
(b) Superdex 200 HR column was equilibrated with 0.01 M sodium acetate, pH 5.0, containing 0.15 M NaCl. The flow rate was 20 ml/h.

peared as a symmetrical peak that coincided with its activity (Fig. 2). A summary of the purification steps is shown in Table I. The enzyme was purified about 230-fold with a yield of 17.7% from the culture filtrate.

Homogeneity and physicochemical properties of the purified α-galactosidase II

The purified α-galactosidase II showed a single protein band on SDS-PAGE (Fig. 3a). The molecular mass of the α-galactosidase II was estimated to be 51–62 kDa by SDS-PAGE, and 60 kDa by gel filtration on a Superdex 200 HR 10/30 column (Fig. 3b). The enzyme interacted with Con A and the size of α-galactosidase II after the treatment with Endo H decreased to 42–47 kDa, however, it still had the reactivity with Con A (data not shown). The isoelectric point of α-galactosidase II was estimated to be 8.5.

Effects of pH and temperature on the activity and stability of α-galactosidase II

As shown in Fig. 4, α-galactosidase II had maximal activity on *p*NP-Gal at pH 3.0–4.0 and 60°C but the enzyme was unstable at low protein concentrations even at room temperature. The addition of BSA to the enzyme solution at the concentration of 0.01% increased its stability considerably (Fig. 4c and d). A high concentration of α-galactosidase II (1 mg/ml) was stable at 4°C and pH 5.0 and the loss of the activity after 6 months was less than 10%.

Substrate specificity

As shown in Fig. 5, α-galactosidase II hydrolyzed melibiose, raffinose, stachyose, and galactomanno-oligosaccharides, such as Gal^3Man_3 and Gal^3Man_4, while α-galactosidase I hardly hydrolyzed Gal^3Man_4. α-Galactosidase I could not act on galactomannans,[2] on the contrary, α-galactosidase II could hydrolyze α-galactosidic linkages of galactomannans and was able to liberate 65% and 85% of the total α-galactosyl residues from guar gum and locust bean gum, respectively (Fig. 6). Locust bean gum and guar gum were insolubilized by the removal of α-galactosyl residues at the time indicated by an arrowhead. The ratios of galactose to mannose of locust bean gum and guar gum were 1/4 and 1/2, respectively. From the glycosyl composition analyses of the polysaccharides at 48 h incubation, the ratios of final products of locust bean gum and guar gum decreased to less than 1/25 and 1/6, respectively.

N-Terminal and internal amino acid sequences

The purified α-galactosidase II and the enzyme digested with lysylendopeptidase were analyzed by SDS-PAGE. After blotting on a PVDF membrane, *N*-terminal amino acid sequences of the purified enzyme and a fragment were identified as I-I-D-P-S-L-A-K-T-P-Q-M-G-W-N-S-W-N-K- and N-V-S-R-P-I-F-F-S-L-, respectively.

Nucleotide sequence and deduced amino acid sequence of the α-galactosidase II cDNA

A mixture of synthetic oligonucleotides [5′-GG(A/G/C)GT(T/C)TACCC(A/T/G/C)ACCTT-3′] that were deduced from the amino acid sequence (P-Q-M-G-W-N) was used as a probe for screening the cDNA. Two positive clones having about 1.2-kbp inserts were obtained in a population of about 60,000 clones by using plaque hybridization. The nucleotide sequence of the cDNA (1240 bp) was identified as shown in Fig. 7. *N*-Terminal and internal amino acid sequence of α-galactosidase II identified by Edman degradation were found in the sequence and underlined. The enzyme probably had 396 amino acid residues composed of 20 amino acid residues of signal sequence and 376 amino acids of mature α-galactosidase II. The molecular mass of the mature enzyme is calculated to be 41,334 Da from the sequence. Five cysteine residues and nine putative *N*-glycosylation sites were found in the sequence.

A consensus polyadenylation signal (AATAAA) is 22 nucleotides upstream of polyadenylic acid. To estimate the size of α-galactosidase II mRNA, an RNA blot hybridization of the purified poly(A^+)RNA with a whole insert DNA as a probe showed the size of the mRNA to be about 1300 nucleotides (data not shown). Thus the missing 5′-region of the mRNA is about 50 nucleotides.

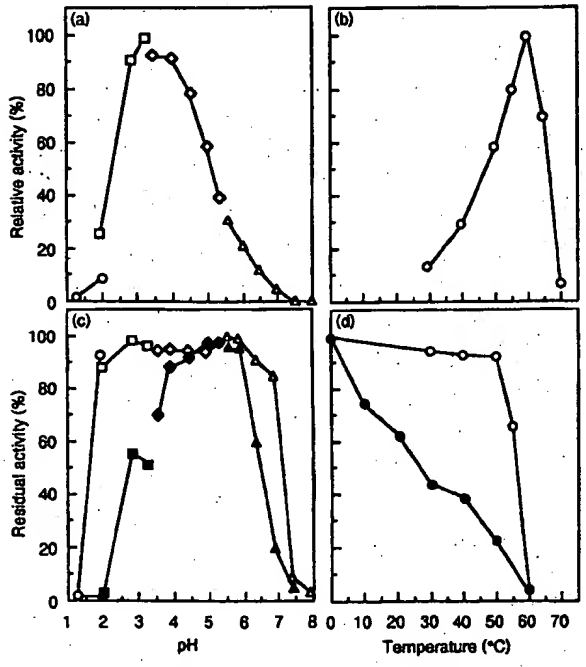

Fig. 4. Effects of pH and Temperature on the Activity and Stability.

(a) The optimum pH was measured at 40°C using buffers as follows: ○, 0.1 M KCl–HCl, pH 1.3–1.9; □, 0.1 M Gly–HCl, pH 2.0–3.3; ◇, 0.1 M sodium acetate, pH 3.6–5.3; △, 0.1 M sodium phosphate, pH 5.5–7.9.
(b) The optimum temperature was measured at pH 5.2 at various temperatures as described in Materials and Methods.
(c) To investigate pH stability, the enzyme was incubated at 30°C and various pHs for 60 min with (open symbol) and without (closed symbol) 0.01% BSA, and the remaining activitites were measured at pH 5.2. Buffers used were the same as in (a).
(d) To examine the thermal stability, the enzyme were incubated at pH 5.0 and various temperatures for 60 min with (○) and without (●) 0.01% BSA, and the remaining activity was measured at 40°C.

Discussion

Two α-galactosidases, I and II, were excreted in the culture medium of *M. vinacea* and were separated by the step of DEAE-Sephadex A-50 chromatography. α-Galactosidase I was adsorbed on the column, and eluted with a linear gradient of 10 to 50 mM of the buffer. This enzyme was further purified by Sephacryl S-300 HR gel filtration and characterized. There was no difference in the physicochemical properties, substrate specificities, or *N*-terminal amino acid sequence between α-galactosidase I and the intracellular enzyme previously reported.[2,15]

As shown in Fig. 3a, purified α-galactosidase II showed a single but broad protein band on SDS–PAGE. This indistinctness is probably due to the heterogeneity of its sugar chain because the enzyme migrated as a broad protein band even when a small amount of the enzyme was used (data not shown). The treatment with Endo H decreased the size of the enzyme but could not eliminate oligosaccharide chains completely because the reactivity with Con A re-

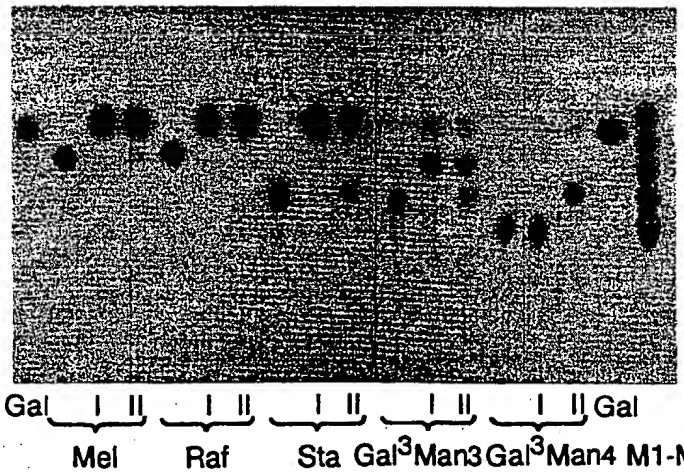

Fig. 5. Actions of α-Galactosidases I and II on Galacto-oligosaccharides and Galactomanno-oligosaccharides.

α-Galactosidase I was purified by the method of Suzuki *et al.*[2] with a slight modification and was homogeneous on SDS–PAGE. The reaction mixture contained 40 μl of 1% substrate, 40 μl of McIlvaine buffer[16] (pH 4.0), and 20 μl of enzyme solution (0.2 units at pH 4.0), which was incubated at 30°C. After 12 h, each of the reaction mixture was boiled for 5 min to stop the reaction. Three μl of the mixture was used for TLC to characterize the hydrolysis products.
Gal, authentic galactose; Mel, melibiose; Raf, raffinose; Sta, stachyose; M_1–M_5, authentic mannose to β-1,4-mannopentaose from top to bottom; I, digested with α-galactosidase I; II, digested with α-galactosidase II.

mained, indicating that α-galactosidase II had *N*-linked oligosaccharide chain(s) and the protein portion of the enzyme was estimated to be less than 42–47 kDa.

Some properties of α-galactosidases I and II were examined and compared in Table II with *p*NP-Gal as a substrate. Although the optimum pH and temperature of these two enzymes were identical, the pH and thermal stabilities of each enzyme were quite different. α-Galactosidase I was stable at neutral and alkaline pHs, while α-galactosidase II was relatively stable at pH 4–6. Although α-galactosidase I was stable up to 50°C at pH 7.0 even at a low protein concentration, α-galactosidase II was unstable above 20°C at pH 5.0. The stability of α-galactosidase II was increased up to 50°C by the addition of 0.01% BSA (Fig. 4).

α-Galactosidase I was quite stable at 4°C and pH 7.0 for more than two years even if the size of the enzyme decreased by degrading its oligosaccharide chains. α-Galactosidase II (1 mg/ml) was stable at 4°C and pH 5.0, if the oligosaccharide chains of the enzyme were unchanged. When the *N*-linked oligosaccharide chains were removed enzymatically with Endo H, α-galactosidase II was aggregated and inactivated completely (unpublished data). This suggests that the sugar chains in α-galactosidase II probably have an important role in constructing its tertiary structure.

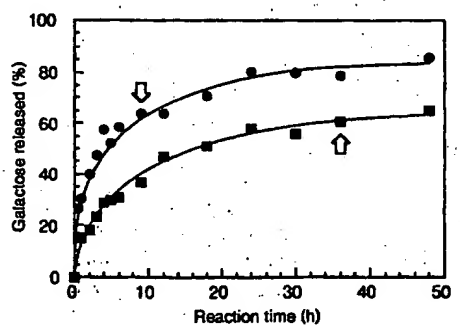

Fig. 6. Actions of α-Galactosidase II on Galactomannans.

The reaction mixture (1 ml) contained 500 μl of substrate (1%), 100 μl of 1 M sodium acetate, pH 4.0, and 2.2 units of enzyme (400 μl) and was incubated at 30°C. Fifty μl of the sample was withdrawn at the indicated times and the galactose liberated was measured by the Somogyi–Nelson technique. Times at which precipitation of the galactomannan occurred are indicated by open arrows. ●, locust bean gum; ■, guar gum.

Molecular forms of α-galactosidases I and II in the solution were quite different. For instance, α-galactosidase II showed its molecular size to be 60 kDa on a Superdex 200 HR column and 51–62 kDa on SDS-PAGE, indicating that the enzyme existed as a monomeric form. On the other hand, α-galactosidase I probably existed as a tetrameric form, because the molecular size of α-galactosidase I was 240 kDa on a Superdex 200 HR column and 50–56 kDa on SDS-PAGE (Table II).

The isoelectric points of α-galactosidases I and II were 5.4 and 8.5, respectively, and it is reasonable that α-galactosidase II passed through the anion exchange column equilibrated at pH 7.0, while α-galactosidase I was adsorbed on the column. These enzymes were easily separated by this step.

α-Galactosidase I cleaved only terminal α-galactosyl residues from the substrates as reported previously,[4] while α-galactosidase II had specificity for all of the substrates tested here. α-Galactosidase II hydrolyzed Gal^3Man_4 more rapidly than Gal^3Man_3 (Fig. 5). Gal^3Man_4 has an α-galactosyl side chain on the third mannose from the reducing end of mannotetraose, thus α-galactosidase II showed preference for the side-chain α-galactosyl residue even though

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   1 AAATGCTCAGCAACAGTCAAATTCTTACTATCCTTGGTCTTTCCGTGGTCCAAGTAATGGGTATCATTGACCCTAGCCTTGCCAAAACACCCCAAATGGGCTGGAACTCTTGGAACAAAT 120
-20  M  L  S  N  T  Q  I  L  T  I  L  G  L  S  V  V  Q  V  M  G  I  I  D  P  S  L  A  K  T  P  Q  M  G  W  N  S  W  N  K  Y  20

 121 ATCAGTGTAATGTGAATGAAACTGTCATCATCAACACTGCCAATGCCATGGTTTCAAGTGGCCTCAAGGACTTAGGATACCATTATATTAACATTGATGATTGCTGGTCATTGCATCAAC 240
  21  Q  C  N  V  [N  L  T]  V  I  I  N  T  A  N  A  M  V  S  S  G  L  K  D  L  G  Y  H  Y  I  N  I  D  D  C  W  S  L  H  Q  R  60

 241 GTGACAATACTACTCAGCGCATTGCGCCTGACCCTACCAAATTCCCAAATGGTATCTCAGGTGTTGCTTCTAAGGTTCATGCACTTGGCCTCAAGTTCGGAATATATTCCGATGCGGGTA 360
  61  D  [N  C  T]  Q  R  I  A  P  D  P  T  K  F  P  N  G  I  S  G  V  A  S  K  V  H  A  L  G  L  K  L  G  I  Y  S  D  A  G  T 100

 361 CCAACACCTGTGCCGGTTATCCAGGATGATATGGATATGAAGCGATCGACGCACAAGCTTTTTCTGATTGGGGAGTTGATTACCTCAAGTACGACAACTGTAACAACCTTGGCTTAGCTG 480
 101  N  I  C  A  G  Y  P  G  S  Y  G  Y  D  A  I  D  A  Q  A  F  S  D  W  G  V  D  Y  L  K  Y  D  N  C  N  N  L  G  L  A  G 140

 481 GAAATGCTACCATTTCCAGCAAACGTTATAAGCGTATGGGAGATGCTCTCAAGAACGTCAGTAGACCCATATTCTTCTCCTTGTGCAGCTGGGGCACGGACGACGTTTGGGACTGGGGTC 600
 141  [F  A  T]  I  S  S  K  R  Y  K  R  M  G  D  A  L  K  [N  V  S]  R  P  I  F  F  S  L  C  S  W  G  T  D  D  V  W  D  W  G  R 180

 601 GATCCATTGGAGGCCAATCATGGCGCATGTCTGGCGACATTTCCGACAACTGGTCTAGCGTTGTGTCCATCACGGGTCAAGCGGTACCTATTGCCAATATCTCAGCCCCTGGTGGATGGA 720
 181  S  I  G  G  Q  S  W  R  M  S  G  D  I  S  D  [N  F  S]  S  V  V  S  I  T  G  Q  E  V  P  I  A  [N  L  S]  A  Q  G  G  W  N 220

 721 ACGATATGGATATGTTGGAAGTCGGTGTACACGATCAAATGACCATCACTGAGTACACTAGCCACTTTTCAATTTGGGCTGCCATGAAAAGCCCTTTGATTCTTGGAAATGATATCACCA 840
 221  D  M  D  M  L  E  V  G  V  H  D  Q  M  T  I  T  E  Y  T  S  H  S  F  I  W  A  A  N  K  S  P  L  I  L  G  N  D  I  T  N 260

 841 ACATGACAAATGATATTAAGAACATCATTACAAACAATGAGGTCATTGCCATCAGTCAAGACTCTCTAGGAGCATCTGTGCAACAAAGATCCATGAAGGGAAATACTCAATTGTTTGCAG 960
 261  [M  N]  N  D  I  K  N  I  I  T  N  N  E  V  I  A  I  S  Q  D  S  L  G  A  S  V  Q  Q  R  W  M  L  G  N  T  Q  L  F  A  G 300

 961 GTCCTTTATCTAAGAATGGCTATGTCTCTCTATTCTTGAATGAGGGTAACTCGACAACTAATATGACCGGTACCTGGTCTGAGATCTTCAATAATCCCAAGGCAAACACACACAAGTCTA1080
 301  P  L  S  K  N  G  Y  V  A  L  F  L  N  E  G  [N  D  S]  T  [N  S]  G  T  W  S  E  I  F  N  N  P  K  A  N  T  H  K  S  I 340

1081 TCGCTGTACGCGATCTCTGGGCACACAAAGACTTGGGCAGATTCCAGGGTAGCATTAGCGTTAGTGTTGAGTCACATGGAGTTCGTATGTTGAAATTCAGTCAACAAGCTTAAAATCCAA1200
 341  A  V  R  D  L  W  A  H  K  D  L  G  R  F  Q  G  S  I  S  V  S  V  E  S  F  G  V  R  M  L  K  F  S  Q  Q  A  *          376

1201 TGCAAATAAACGTATGATTTACCCACAAAAAAAAAAAAAAAAA                                                                    1243
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Fig. 7. The Nucleotide Sequence of the cDNA and Deduced Amino Acid Sequence.

Nucleotides were numbered beginning with the first residue of the cDNA. Amino acid residues were numbered from the *N*-terminus of the mature α-galactosidase II, which is indicated with an arrowhead. The termination codon is indicated by an asterisk. Putative *N*-glycosylation sites are indicated in a shaded box, cysteine residues are double-underlined, and amino acid sequence of α-galactosidase II as found by Edman degradation is underlined.

the enzyme acted on the terminal one attached to the non-reducing end mannose.

Kaneko *et al.* classified microbial α-galactosidases into two groups based on the substrate specificity on galactomanno-oligosaccharides.[6] The first group consists of *M. vinacea* α-galactosidase I and the enzyme belonging to this group tore off the α-galactosyl residue attached to the non-reducing end mannose of manno-oligosaccharides such as Gal^3Man_3. The second group consists of α-galactosidases from *Aspergillus niger* 5–16 and *Penicillium purpurogenum*, which acted only on the side-chain α-galactosyl residue on manno-oligosaccharides such as Gal^3Man_4.[7] α-Galactosidase II obtained from *M. vinacea* hydrolyzed both terminal and side-chain α-galactosidic linkages on the substrate.

Table II. Some Properties of α-Galactosidases I and II

	α-Galactosidase I	α-Galactosidase II
Molecular mass		
SDS–PAGE	50–56 kDa	51–62 kDa
Gel filtration	240 kDa	60 kDa
Oligosaccharide chain	+	+
Isoelectric point	5.4	8.5
Optimum pH	3.0–4.0	3.0–4.0
Optimum temperature	60°C	60°C
pH stability	6.0~	4.0–6.0 2.0–7.0*
Heat stability	~50°C	~20°C ~50°C*

* In the presence of 0.01% BSA.

The high viscosities of guar gum and locust bean gum are extensively used in some industries including the food industry. Galactomannans are also good promoters of gelling when mixed with some polysaccharides such as carrageenan, and xanthan gum.[22] Locust bean gum (Gal/Man = 1/4) interacts more strongly with gelling polysaccharides than guar gum (Gal/Man = 1/2). Removal of galactose moieties from guar gum to generate a modified galactomannan by α-galactosidase will yield a polysaccharide with functional properties comparable with those of locust bean gum. Dey *et al.*[22] demonstrated that relatively few α-galactosidases can cleave α-galactosyl residues from galactomannan. α-Galactosidases from coffee,[23] guar,[24] and *Penicillium ochlochloron*[22] have been reported to eliminate galactose from galactomanan, however, these enzymes needed to be separated from the β-mannanase activity in the crude enzyme completely. The α-galactosidase activity of *M. vinacea* was induced by the addition of lactose,[25] and the α-galactosidase without β-mannanase activity was obtained from the culture filtrate by simple purification steps. Although α-galactosidase I hardly acted on the galactomannan as reported previously,[2] α-galactosidase II could hydrolyze galactomannan effectively and decreased the galactose content of the polymer as shown in Fig. 6. Thus α-galactosidase II is a suitable enzyme for modifying the Gal/Man ratio of galactomannans.

A comparison of amino acid sequences of α-galactosidases from *M. viancea* (α-galactosidase I),[15] *Saccharomyces carlsbergensis*,[10] *Cyamopsis tetragonoloba* (guar),[9] coffee,[12] human,[8] and *A. niger*,[11] with α-galactosidase II is depicted in Fig. 8. It shows that there is a considerable

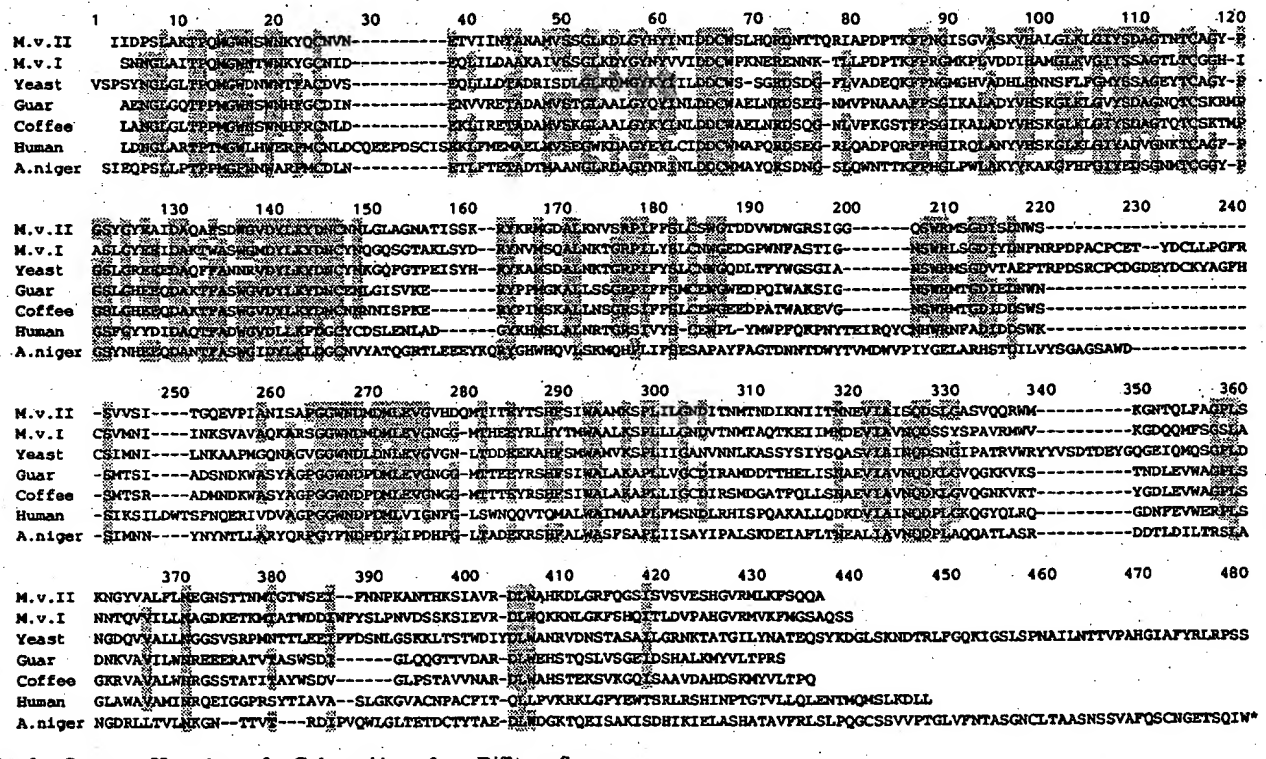

Fig. 8. Sequence Homology of α-Galactosidases from Different Sources.

The sequences were aligned for optimal sequence similarity by using the program GENETYX (Software Development) and manual arrangement. The numbers above the sequence indicate the relative position of each amino acid sequence. The sequences of *A. niger* α-galactosidase are truncated at the *C*-terminus, indicated by an asterisk. Identical amino acid residues, five out of seven or more at the same position, are in the shaded box. M.v.II, *M. vinacea* α-galactosidase II; M.v.I, *M. vinacea* α-galactosidase I; yeast, *S. carlsbergensis* α-galactosidase.

degree of similarity. The sequence identity of α-galactosidase II with the enzymes from *M. vinacea* (α-galactosidase I), *S. carlsbergensis*, *C. tetragonoloba*, coffee, human and *A. niger* were 49%, 42%, 45%, 46%, 39%, and 31% respectively. On the other hand, procaryotic α-galactosidases such as those of *E. coli*[13] and *Streptococcus mutans*[14] show little similarity with eukaryotic enzymes. Although α-galactosidases II and I showed the highest similarity among α-galactosidases, α-galactosidase II showed relatively high similarity with the enzymes from guar and coffee, which could act on the galactomannan.[23,24] All of the five cysteine residues in α-galactosidase II were present at the identical positions to α-galactosidase I. The biggest difference between the two was the 20 amino acid residue insertion around the 200th residue of α-galactosidase I. This insertion was also observed in the sequence of *S. carlsbergensis* α-galactosidase.

Ishii *et al.* studied α-galactosidase activity by using chimeric proteins comprising human α-galactosidase and α-*N*-acetylgalactosaminidase.[26] Their data suggest that two regions from the 15th to 59th residue and from the 263rd to 291st residue of human α-galactosidase contribute to the α-galactosidic cleavage and substrate recognitions (numbering is based on Fig. 8). These regions contain some conserved amino acid residues among all α-galactosidases. Chemical modification study has indicated the presence of two carboxyl groups at or near the active site of α-galactosidase.[27] Thus it may be concluded that carboxyl residues located in such homologous regions serve as an active site.

Some amino acid residues that are critical for the enzymatic activity were identified by the studies on human[28-31] and coffee[32] α-galactosidases, however, little is known about the structure-function relationship of α-galactosidases.

Although α-galactosidases I and II from *M. vinacea* share high sequence similarity, some of their properties, such as substrate specificity and pH stability, are distinct. Expression and mutagenesis study of cDNAs encoding these α-galactosidases in *S. cerevisiae* are now in progress. The study will provide some information about the structure-function relationship of α-galactosidase.

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